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AN OVERVIEW OF PLATELET STRUCTURAL PHYSIOLOGY

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Abstract

Marion Barnhart and her colleagues used light, phase contrast and scanning electron microscopy to provide a clear picture of platelet surface changes developing in response to aggregating agents. This review, in honor of Marion and her work, has sought to expand the horizon provided through study of surface alterations by peeling back the membrane of the platelet to reveal the dynamic world within. A cytoskeleton consisting of a circumferential microtubule and submembrane actin filaments supports the discoid shape of the resting cell. Following exposure to aggregating agents in suspension, to foreign surfaces or denuded blood vessels and to fibrin strands during clot retraction, the state of assembly and organization of actin and location of the microtubule undergo dramatic alterations. There are differences in the cytoskeletal organization of platelets activated in suspension or on surfaces, during spreading on damaged blood vessels or in the process of clot retraction; yet, the similarities are far more impressive than the differences. The ability to assemble its cytoskeleton and adapt it to a variety of conditions requiring the exercise of contractile force makes the platelet a unique form of muscle cell.

KEY WORDS: Platelet, Open canalicular system, dense tubular system, Circumferential microtubule, Alpha granules, Dense bodies, Lysosomes, Glycocalyx, Cytoskeleton, Dendritic forms, Spread forms.

Introduction

The morphological sciences have played an important role in the development of our current state of knowledge regarding platelet structure, function and pathology.[41] Microscopists were the first to recognize platelets and characterize their role in blood coagulation and hemostasis.[6,12,23,37] Biochemistry, physiology, immunology and, most recently, molecular biology[10,13,18] have become increasingly important in studies of platelets.[46] However, they have not replaced the morphological approach which continues to make important contributions.[5,11,55,68]

Marion Barnhart realized the value of microscopy in platelet studies. She and her colleagues provided an excellent, three-dimensional view of platelet responses to chemical stimuli and the influence of inhibitors. Her interests were focused on the structural physiology of platelets according to three different morphologic approaches. Transmission electron microscopy of whole mount preparations permitted observations of platelet profiles and quantitation of the platelet responses in their natural environment of blood and effects of various additives. Scanning electron microscopy revealed in striking three-dimensional relief the fine detail of platelet aggregation. Finally, light microscopy supplemented with Normarski optics provided a 3-D visualization of living platelets. The three different morphologic techniques provided a balanced approach to the study of platelet surfaces. Marion excelled in the use of SEM techniques which she presented in several publications and reviewed in 1972.[2] Her findings have stood the test of time, and remain as a testament to her scientific contributions.

The present report is part of a symposium organized to honor Marion Barnhart. Marion was a friend of mine, and I am pleased to participate in this recognition of the person and the scientist. The approach taken in our laboratory has differed from that of Marion and her colleagues. However, her work serves as an excellent background for this overview of platelet structural physiology.

Prologue

Light microscopic studies revealed existence of the blood platelet and some features of its deceptively simple morphology.[6,12,23,37] Each cell consisted of a variable number of organelles randomly dispersed in a water-clear cytoplasm enclosed by a plasma membrane. The small size of the cell and relatively featureless appearance had contributed to the long delay in its discovery as a normal constituent of blood. However, once existence of the platelet was established, its simple appearance and characteristic discoid form in the resting state provided a convenient means for easy recognition.

Unfortunately, the limited resolution of the light microscope did not permit evaluation of fine details of platelet anatomy. Most of our knowledge concerning platelet structure-function relationships has come from examination of the cells in the electron microscope;[7,11,55,68,72] however, one ultrastructural approach has not been sufficient. Many types of electron microscopes, combined with ultrastructural cytochemistry and immunocytochemistry, have been required to elucidate details of platelet structural physiology.[55] The observations of many colleagues,[17,26,42] together with the excellent work of M.I. Barnhart, have provided the picture of platelet structure and function we see today.

Resting Platelets

In order to appreciate the changes characteristic of the platelet response to stimulation, it was essential to develop a clear understanding of the resting platelet. The scanning electron microscope (SEM) made this possible. It provided a nearly three-dimensional image of the discoid cell at high magnification.[2] The platelet surface is relatively smooth when viewed by SEM, like that of the erythrocyte, but is considerably smaller and lacks the biconcave appearance typical of the red blood cell (Fig. 1). A variable number of miniscule openings, usually grouped in small areas rather than distributed at random, are apparent on the surface of the resting platelet. They represent pores through which channels of the open canalicular system communicate with the exterior. Ruffles, spikes and lamellapodia present on lymphocytes, monocytes and granulocytes are not apparent on discoid platelets.

Thin sections of resting platelets provide information on their structural organization (Fig. 2). In order to relate structure to function, the anatomy of the platelet has been divided into several zones.[54,63] The peripheral zone consists of membranes and closely associated structures providing the surface of the platelet and walls of the tortuous channels making up the surface-connected open canalicular system (OCS) (Fig. 3). An exterior coat, or glycocalyx, rich in glycoproteins,[40] provides the outermost covering of the peripheral zone. Its chemical constituents provide the receptors for stimuli triggering platelet activation and the substrates for adhesion-aggregation reactions. The middle layer of the peripheral zone is a typical unit membrane. It is rich in asymmetrically distributed phospholipids that provide an essential surface for interaction with coagulant proteins. The area lying just inside the unit membrane represents the third component of the peripheral zone. It is closely linked to the unit membrane and the exterior coat through trans-membrane portions of GPI_b (missing in Bernard Soulier syndrome)[36] and $GPII_b$-III_a (missing in thrombasthenia).[38] The submembrane area translates signals received on the outside surface into chemical messages and physical alterations required for platelet activation.

The sol-gel zone is the matrix of the platelet cytoplasm. It contains several fiber systems in various states of polymerization that support the discoid shape of unaltered platelets and provide a contractile system involved in shape change, pseudopod extension, internal contraction and secretion. Elements of the contractile system appear to be major components since they constitute approximately 25-30% of the total platelet protein.[16] Masses, as well as discrete particles of glycogen, are distributed in the sol-gel matrix.

The organelle zone consists of granules, electron dense bodies, peroxisomes, lysosomes and mitochondria randomly dispersed in the cytoplasm (Fig. 2). It serves in metabolic processes and for the storage of enzymes, non-metabolic adenine nucleotides, serotonin, a variety of protein constituents, and calcium destined for secretion.

Membrane systems represent a special zone in platelet anatomy. The dense tubular system (DTS) (Fig. 4) has been shown to be the site where calcium, important for triggering contractile events, is sequestered.[65] Also, it is the site where enzymes involved in prostaglandin synthesis are localized.[19] The OCS provides access to the interior for plasma-borne substances and an egress route for products of the release reaction. Together with elements of the dense tubular system, channels of the OCS form specialized membrane complexes (Figs. 2, 4) which closely resemble the relationship of transverse tubules and sarcotubules in embryonic muscle cells. Highly regulated interactions of structural constituents in the four major zones facilitate the normal activity of platelets in hemostasis.

Platelet Cytoskeleton

Methods used to fix, dehydrate and embed platelets for study in thin sections tend to destroy or distort critical elements of the platelet cytoskeleton.[32] Actin filaments are often lost in the maze of other proteins making up the matrix of the platelet. Elements of the cytoskeleton can become visible in platelets allowed to interact with the surface of carbon-stabilized, formvar-coated grids and negatively stained with silicotungstate.[35] Discoid platelets prepared in this manner may have a lens-like effect on the conventional electron beam, and appear transparent, except for the dense bodies which are inherently electron opaque (Fig. 5). Exposure to critical

point drying allows visualization of substructure in discoid cells, but the procedure, even when preceded by fixation in glutaraldehyde, results in distortion of subcellular anatomy.[28]

If platelets are allowed to spread on grid surfaces, negative stains will penetrate the cell and outline elements of the cytoskeleton (Fig. 6). However, the process of spreading and retention of lipid constituents of the cell surface, canalicular systems and enclosing organelles cause considerable distortion of the cytoskeleton. The circumferential microtubule (MT) coils are somewhat distorted by the whole mount drying procedures, though they remain reasonably intact. Microfilamentous elements are illuminated by the negative stain, but their organization may be considerably deranged during preparation.

These difficulties can be circumvented by simultaneous fixation with glutaraldehyde and extraction with the detergent, triton X-100.[24,44] The procedure removes the surface bilayer, OCS, DTS and membranes enclosing all organelles. Detergent resistant residues remain in the cell (Fig. 7). Only a small fraction of the actin molecules are assembled into long filaments in resting platelets.[58,69] Thus, the major element serving as the peripheral border of the discoid cell is the circumferential microtubule. The cytoplasm is packed with an irregular meshwork of fibrillar material. Granule residues are evident as electron opaque shadows in the detergent extracted cytoplasm.

The cytoskeleton of suspended platelets can be preserved for demonstration in thin sections if special care is taken to block the destructive effects of osmic acid. Lysine,[8] or a combination of lysine and phalloidin[15] protect actin filaments from damage during glutaraldehyde-osmium fixation and dehydration in alcohols. The Triton X-100 added to the initial step of fixation removes all of the cell membranes, leaving behind the detergent resistant residues (Fig. 8). Coiled circumferential microtubules appear to serve as the peripheral margins of the extracted cells cut in the equatorial plane, but cross sections reveal a layer of short actin filaments woven together outside the circumferential MT.[8,15] These are the submembrane filaments described many years ago,[61] and are the major contribution of actin to the membrane cytoskeleton of resting platelets. A few actin filaments which have resisted detergent extraction and osmium fixation are present in the cytoplasm of discoid platelets, but the primary localization is at the periphery just under the cell margin. Cytoskeletons prepared from platelets in suspension are virtually identical in appearance to those developed from discoid platelets attached to grids and then exposed to simultaneous detergent extraction and fixation.[8,15,69]

Platelet Shape Change

Exposure of platelets to aggregating agents, foreign surfaces or denuded blood vessels causes rapid activation. The cells lose their resting discoid form, become relatively spherical and extend spiky and bulky pseudopods. Shape change is one of the most dramatic features of the platelet response. The extent of shape alteration is proportional to the nature and concentration of the stimulus. It may precede, accompany or occur after development of platelet stickiness. Internal transformation always accompanies shape change. Irreversible aggregation, clot retraction and hemostatic plug formation are invariably associated with loss of platelet discoid form. Thus shape change is a critical feature of platelet structural physiology.

Dendritic Forms

Activation of discoid platelets in suspension results in transformation to the spiny spheres described by Zucker and Borelli[73] (Figs. 9, 10). Observations on living platelets have shown that the process is dynamic, involving extension and retraction of pseudopods in a repetitive manner.[1] Activation may lead to irreversible physical changes, or, if the stimulus is terminated early, can be reversed completely.

Reorganization of platelet internal structure following exposure of the cells to potent aggregating agents has been followed in considerable detail.[63,67] Granules randomly dispersed in resting cells become concentrated in cell centers (Fig. 11). The circumferential MT coils supporting the shape of discoid cells are constricted into tight rings around the centrally associated organelles. Actin microfilaments are also present with constricted MT rings, but are difficult to recognize in thin sections due to damage caused by osmic acid.

Higher concentrations of physiologic agonists cause more extensive internal transformation (Fig. 12). Channels of the open canalicular system become dilated and often contain substances secreted from granules. Actomyosin is concentrated in the cell centers forming a dense spot of contractile gel. Constricted microtubule rings are also present, but may appear twisted, crushed or fractured by the contracted actomyosin. The contents of most granules have been secreted by such platelets, and they are at an irreversible stage of transformation. Individual actin filaments remain obscure as a result of the influence of osmic acid.

Lysine and phalloidin, together with a low concentration of osmic acid, protect the cytoskeleton of activated platelets in suspension during detergent extraction and fixation (Fig. 13).[8,15] The cytoskeleton of activated platelets consists of a peripheral layer of submembrane actin filaments following the contour of the shape change produced by exposure to an aggregating agent (Fig. 14). A variable number of actin filaments in the cytoplasm extend into pseudopods. The rest of the actin filaments form a concentric mass with constricted rings of MT in the cell centers. The appearance is nearly identical to that observed in detergent extracted whole mounts of surface activated platelets.

Spread Forms

Interaction of platelets with foreign surfaces results in changes similar to those observed in suspended cells following activation.[31,34] The cells become irregular and extend pseudopods in many directions (Fig. 15).

Figure 1. Discoid platelet. Surface features of resting platelets are revealed to advantage in the scanning electron microscope (SEM). The resting platelet has a discoid appearance and relatively smooth contours. Indentations (▲) probably represent communications between channels of the open canalicular system and the cell wall. Bar = 1 μm.

Figure 2. Discoid platelet. Platelet from sample of C-PRP fixed in glutaraldehyde and osmic acid. The cell has been sectioned in the equatorial plane. A circumferential microtubule (MT) lying just under the surface membrane supports the discoid form. Granules (G), occasional mitochondria (M), a few dense bodies (DB), and glycogen particles (Gly) are irregularly distributed in the cytoplasm. Channels of the open canalicular systems (OCS) and dense tubular system (DTS) are randomly dispersed. In some cytoplasmic areas elements of the OCS and DTS are interwoven to form membrane complexes (MC). Bar = 1 μm.

Figure 3. The channel system. Cross section of discoid platelet from sample of C-PRP stained with tannic acid during glutaraldehyde-osmium fixation. Circular profiles of microtubules (MT) supporting platelet discoid shape are evident at each pole of the cell. Tannic acid has acted as a mordant to deposit osmic acid in the exterior coat covering the exposed surface and channels of the open canalicular system (OCS). The OCS forms a system of interconnected channels stretching from one side of the cell to the other. Granules (G) and masses or single particles of glycogen (Gly) are dispersed in the cytoplasm. Bar = 1 μm.

Figure 4. The channel systems. A resting platelet from a sample of C-PRP incubated for peroxidase activity. The osmium black enzyme reaction product is selectively localized to channels of the dense tubular system (DTS). Most channels of the DTS are dispersed at random. However, one channel is usually found in close association with the circumferential microtubule and many develop close relationships with elements of the open canalicular system (OCS) resulting in formation of membrane complexes (MC). Bar = 1 μm.

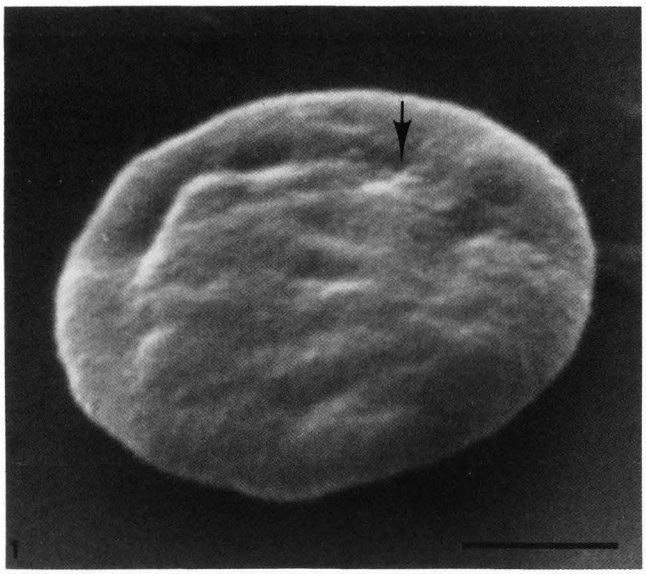
Figure 5. Platelet whole mount. A drop of C-PRP was placed on a formvar-coated, carbon stabilized grid for 1 minute, excess fluid drawn off with filter paper and then dried in air. The unstained platelet is more transparent to the electron beam than the surrounding formvar. However, dense bodies (DB) are inherently electron opaque and are the only internal platelet structures visible under these conditions. Bar = 1 μm.

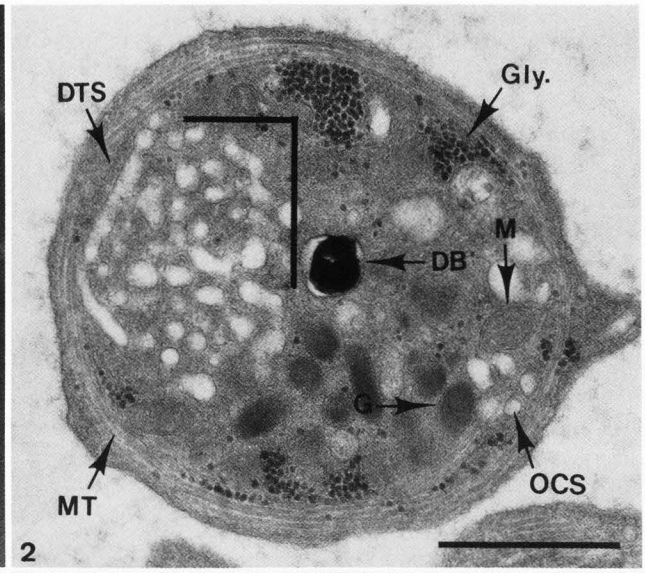
Figure 6. Negatively stained whole mount. Platelet prepared on a grid as described in the previous illustration, and then negatively stained with phosphotungstic acid. The stain outlines internal structures including the circumferential microtubule (MT), granules (G) and dense bodies (DB). Elements of the channel system form a spaghetti-like membranous maze in the background. Bar = 1 μm.

Contact with the surface appears tenuous at an early stage, but quickly changes. Platelets appear to sink into the surface and spread out on it. Organelles remain centrally concentrated. Pseudopods, initially extended in all directions, now interact almost exclusively with the surface. Spaces between pseudopods are filled in, resulting in an appearance resembling a fried egg. The surface of the platelet away from the elevated central area is smooth and featureless (Fig. 16).

Platelets interacting with surfaces develop the same internal transformation as cells activated in suspension (Fig. 17). Concentration of organelles in cell centers and enclosure within rings of microtubules can be identified in whole mounts,[58] but the process is more clearly demonstrated in sections[67] (Fig. 11). On the other hand, features of the cytoskeleton obscured in fixed and sectioned material are beautifully revealed in whole mounts, particularly after detergent extraction.[28,31,34,58] Removal of the membranes allows the interior of platelets to be studied by SEM. Fibrous and filamentous proteins make up a peripheral weave responsible for the spreading process. Filaments also form concentric masses near cell centers. Higher magnification reveals interaction of actin bundles and microtubules. Small attachment plaques present in the cytoplasm suggest sites of transmembrane interaction with the substrate (Fig. 18).

TEM of simultaneously fixed and detergent extracted platelets at various intervals during spreading has also revealed fundamental aspects of cytoskeletal reorganization (Figs. 19, 20). The most impressive feature is the assembly of actin molecules into filaments. Areas of the cytoplasm spreading beyond the MT coils and extending into pseudopods are filled with actin filaments associated in parallel bundles. Organization of actin into parallel associations that impinge on the cell surface may provide the force necessary to drive the surface membrane out into pseudopodial extensions. During conversion of discoid platelets into dendritic forms and assembly of actin molecules into filaments, the circumferential MT are constricted into tight rings in the central zone of the cell. Forces generated at advanced stages of internal contraction may twist or fracture the rings, resulting in extension of single MT toward the platelet periphery and crushing of tubular elements into

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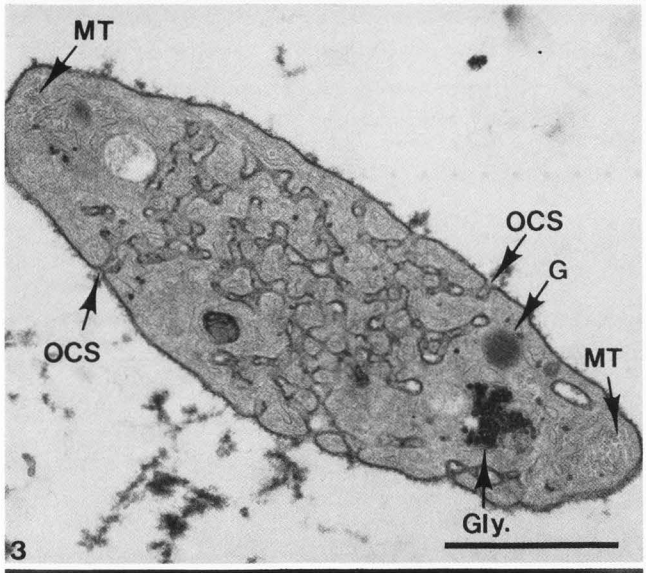
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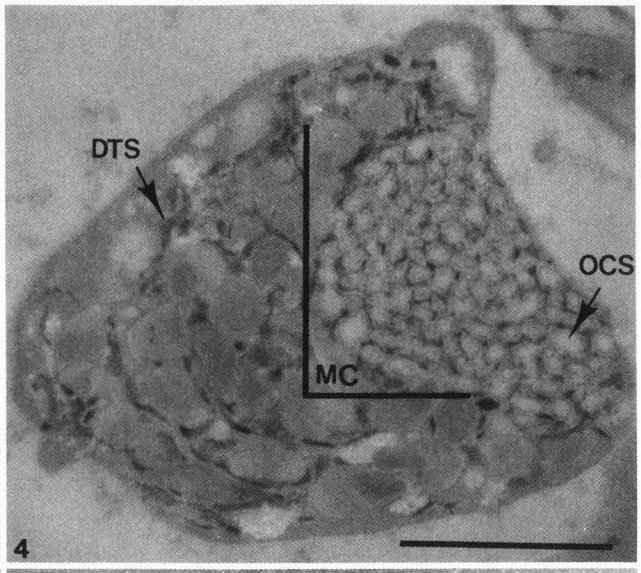
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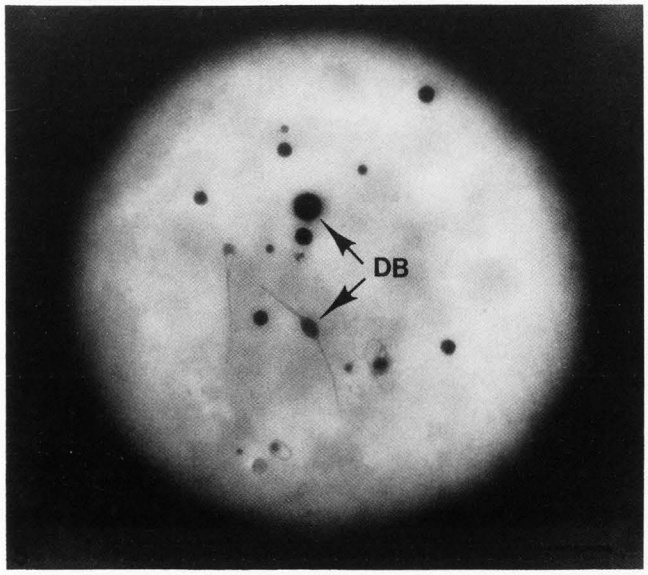
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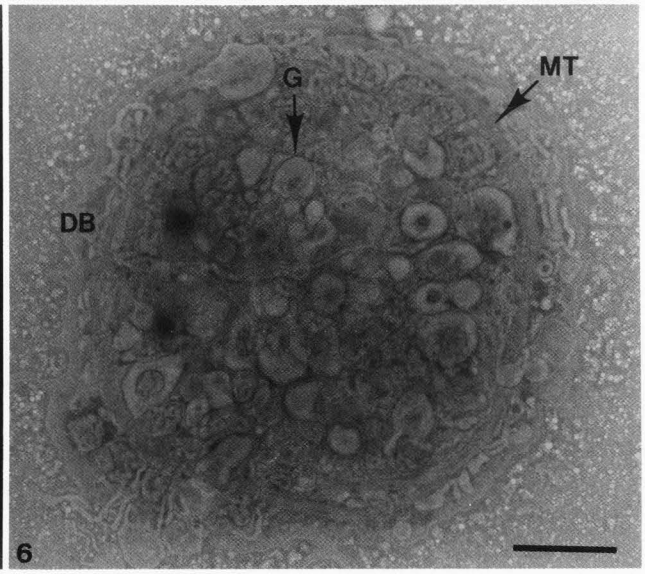
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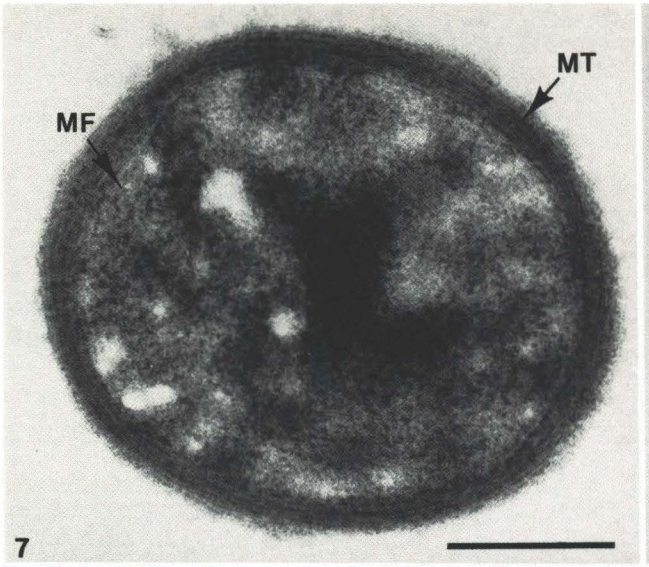
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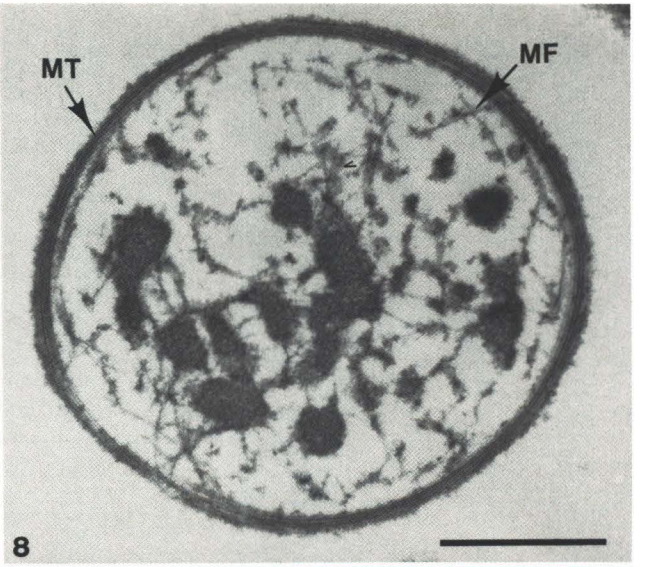
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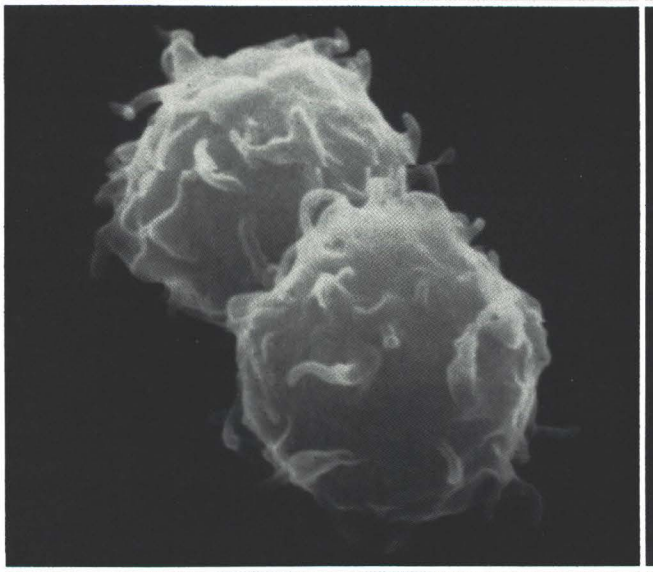

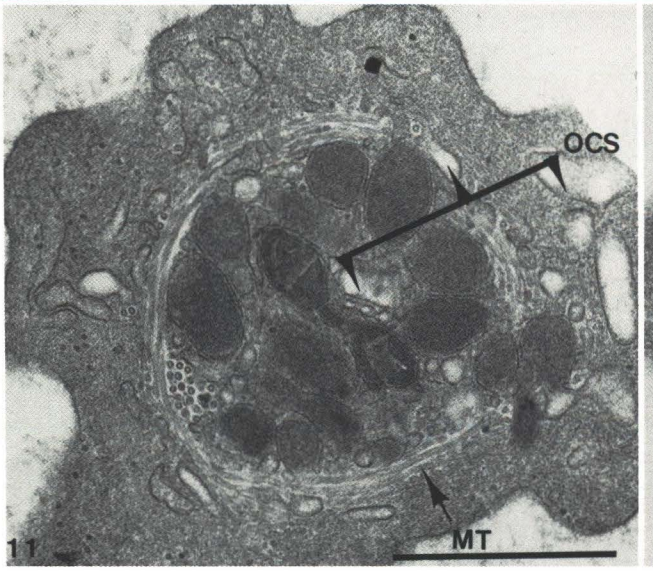
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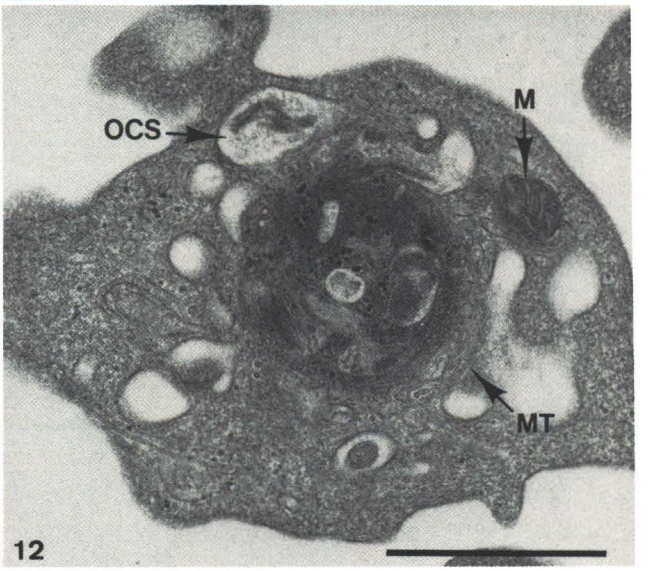
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Figure 7. Whole mount cytoskeleton. Discoid platelet. A platelet allowed to interact briefly with a carbon-coated grid before simultaneous detergent extraction with Triton^{R} X-100 and fixation in glutaraldehyde. All membranes and other lipid-containing structures have been extracted. Only detergent-resistant proteins remain. The circumferential microtubule (MT), together with submembrane microfilaments (MF), forms the border of the cell. Cytoplasm contains vague shadows of granules and only a few filaments which may have assembled during contact with the surface. Bar = 1 μm.

Figure 8. Suspension cytoskeleton. Thin section of a platelet fixed after detergent extraction in the presence of lysine and phalloidin. The microtubule (MT) is well preserved. A rough amorphous coat is evident on its outer surface. Remnants of alpha granules are suspended in a matrix of microfilaments (MF) resistant to detergent extraction. Bar = 1 μm.

Figure 9. Shape change. SEM of platelets from sample of C-PRP fixed 10 seconds following exposure to ADP. The cells have lost their discoid shape, become relatively spherical and extended short pseudopods. Bar = 1 μm.

Figure 10. Shape change. Platelets from sample of C-PRP fixed 30 seconds after exposure to ADP. The cells have lost their discoid form, extending long pseudopods and are adhering to each other. Bar = 1 μm.

Figure 11. Platelet from sample of C-PRP combined with 0.2 U of thrombin/ml, inverted once and allowed to stand at 37°C until fixed two minutes later. The cell has lost its discoid form, became irregular and developed internal transformation. Granules are concentrated in the cell center and enclosed within a constricted ring of microtubules (MT) and microfilaments. Elements of the open canalicular system (OCS) are located throughout the cell. Bar = 1 μm.

Figure 12. Platelet from sample of washed platelets exposed to 5 U of thrombin/ml in the presence of EDTA. Granules are concentrated in a dense mass of contractile gel and constricted microtubules (MT). Mitochondria (M) are seldom incorporated into the central mass. Fibrin-like material is apparent in channels of the open canalicular system (OCS). Bar = 1 μm.

the central mass of contractile gel.[63,67] (Fig. 12)

Development of dendritic forms resulted from assembly of actin filaments and their organization into parallel bundles. Spreading involves a similar process in which assembling actin filaments join with submembrane filaments to expand the margin of the cell (Figs. 21, 22)[27,69] Expansion of the border and thinning of the cytoplasm result in the fully spread platelet. Actin filaments also form parallel bundles resembling stress fibers in the cytoplasm of spread platelets or concentric masses around centrally constricted rings of microtubules. The cytoskeleton of surface activated platelets viewed by SEM or TEM reveals aspects of the cytoskeleton which are slightly different from those observed in thin sections of cytoskeletons prepared from platelets activated in suspension and examined in thin sections. However, the similarities are far greater than the differences.

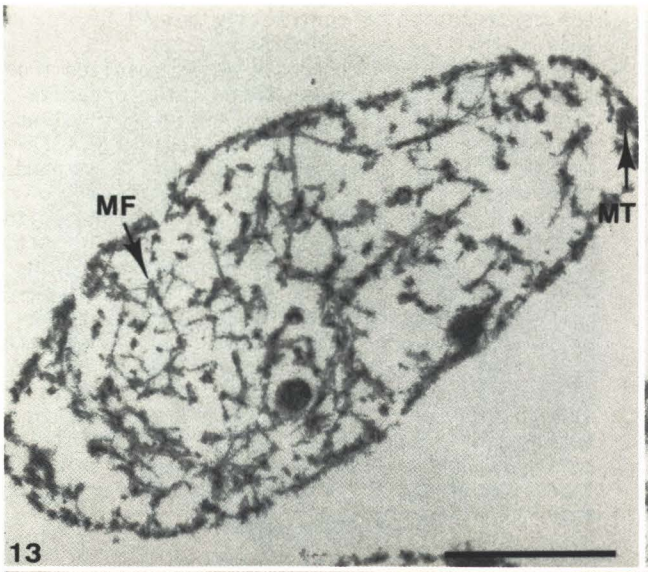
Platelet Secretion

Human platelets are secretory cells. Three different types of secretory organelles, including alpha granules, dense bodies and lysosomes, are present and randomly distributed in resting platelets. The process of their secretion has been a subject of interest since the release reaction was first described.[22] Early studies identified a system of tortuous, interconnected canaliculi in resting platelets which appeared to serve as conduits for the discharge of storage granule products during secretion.[62] A similar conclusion regarding the role of the open canalicular system (OCS) was reached in a study that demonstrated fibrin strands in the channel system of platelets activated by thrombin in the presence of EDTA.[25]

More recent investigations, however, have questioned the process of platelet secretion proposed in the early studies.[14, 21, 39, 43, 47] Ginsberg et al[21, 47] used immunofluorescence with monoclonal antibodies to granule products and electron microscopy to follow the secretory process in thrombin-stimulated platelets. Other agents, such as phorbol myristate acetate[53] and the synthetic diglyceride, oleoylacetyl glycerol,[20] that cause secretion without platelet internal transformation, also stimulate vacuole formation in platelet cytoplasm.

Investigations of bovine platelets have also weakened arguments favoring a central role for the OCS in platelet secretion. Bovine platelets have been shown to secrete the same granule-based products as human cells.[33] However, Zucker-Franklin et al[75] have shown that cattle platelets are virtually devoid of an open canalicular system. Because of the basic similarity in the secretory response of human and bovine cells, they questioned the role of the OCS in release reaction of either cell type.

In recent studies we have examined the release reaction in bovine platelets and re-evaluated the secretory pathway in human cells.[57,71] Tannic acid, often used as an electron dense stain, was employed to delineate the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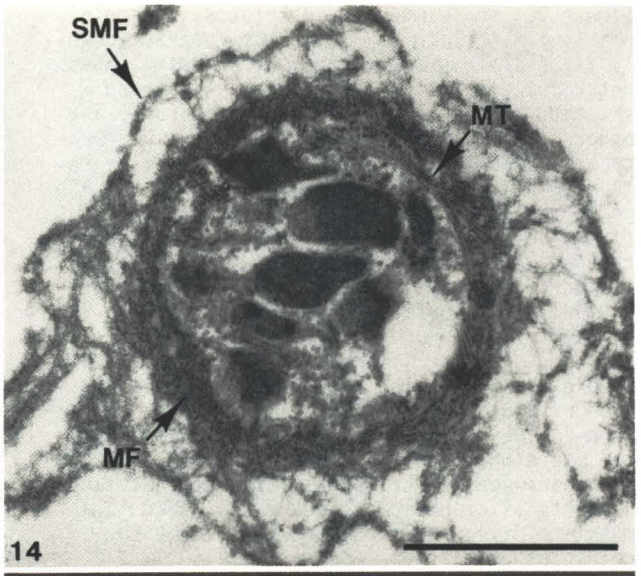
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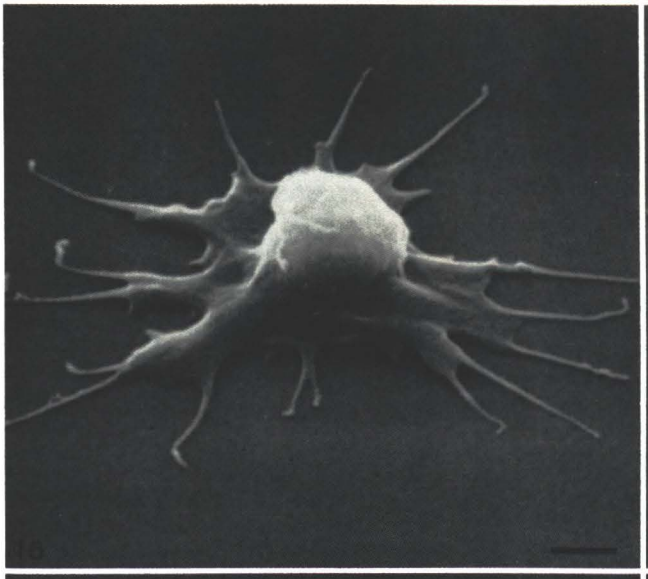
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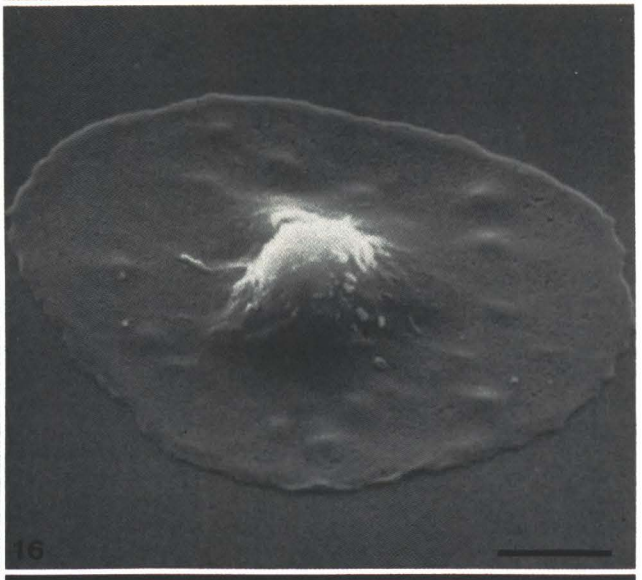
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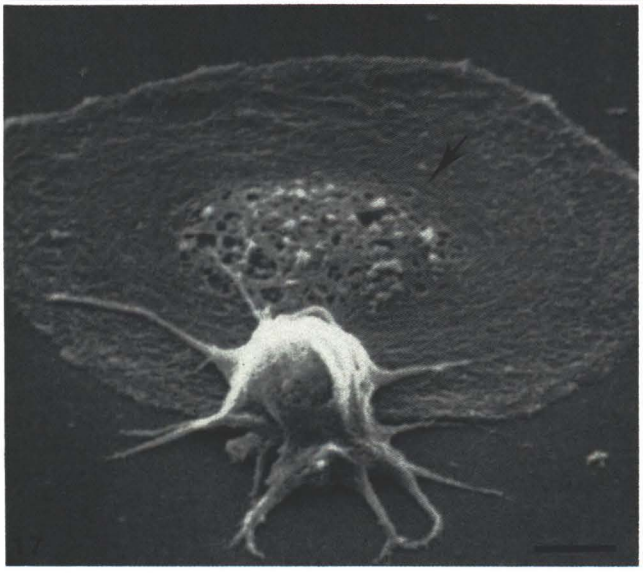

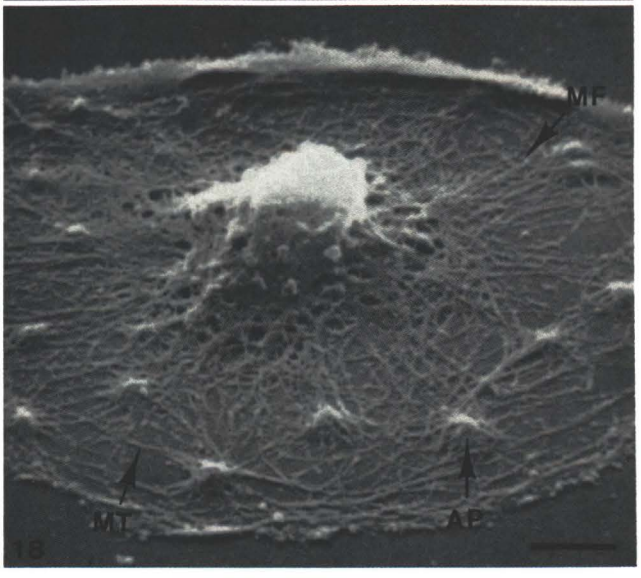
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Figure 13. Suspension cytoskeleton. Cross-section of a discoid platelet cytoskeleton prepared as in Fig. 8. The cell is from a sample of C-PRP treated with taxol (10^{-4} M) before extraction. Microtubule (MT) profiles are evident at the polar ends of the cell. A meshwork of the microfilaments (MF) replaces the cytoplasmic matrix. Although the surface membrane is gone a fine amorphous layer remains in its place. Bar = 1 μm.

Figure 14. Suspension cytoskeleton of activated platelet. The cell is from a sample of washed platelets activated by thrombin, then fixed in solutions containing lysine and phalloidin and finally exposed to a low concentration of osmic acid. Detergent extraction has removed all of the cell membranes. Polymerized actin is concentrated as a layer of submembrane filaments (SMF) at the cell margin and in pseudopods. A ring of actin microfilaments (MF) encloses centrally concentrated organelles. Coils of the circumferential microtubule (MT) are present in the ring of microfilaments. Bar = 1 μm.

Figure 15. Activated platelet. Platelet from a drop of C-PRP allowed to interact with a carbon coated surface for 20 minutes before fixation. The cell has lost its discoid shape and becomes relatively spherical. Portions of the surface in contact with the grid have extended long pseudopods. This is a typical dendritic form. Bar = 1 μm.

Figure 16. Activated spread platelet. Interaction with the surface has caused this cell to spread into a thin film. Centrally concentrated organelles form a hump in the cell center. Bar = 1 μm.

Figure 17. Spread platelet cytoskeleton. A dendritic platelet at the edge of the spread cell does not appear drastically altered by exposure to the detergent fixation sequence. Actin filaments are arranged in concentric layers (▲) around residues of extracted granules in the platelet center. Bar = 1 μm.

Figure 18. Spread platelet cytoskeleton. Actin microfilaments (MF) form the border of this cell and are randomly and radially arranged in the cytoplasm. Coils of the circumferential microtubule (MT) are interspersed with actin bundles. Lumps of detergent resistant material from which actin filaments radiate are adhesion plaques (AP). Bar = 1 μm.

process of secretion. Tannic acid in our preliminary investigation was found to precipitate fibrinogen and selectively deposit osmic acid on fibrinogen and fibrin.

Samples of citrate platelet-rich plasma (C-PRP) and washed human platelets stimulated by thrombin in the presence of ethylenediamine tetracetic acid (EDTA) develop dramatic changes in their morphology.[68] The cells lose their lentiform appearance, become irregular in form, extend numerous pseudopods and aggregate in the absence of EDTA. Platelet organelles become concentrated in cell centers and enclosed within rings of constricted microtubules (Fig. 11). Higher concentrations of thrombin cause rapid discharge of granule contents and reduction in their number. As a result, dense spots of actomyosin in which centrally concentrated organelles are enclosed in less activated platelets, appear more prominent in strongly stimulated cells (Fig. 12). Tannic acid stained platelet aggregates from C-PRP were identical in appearance to unstained control aggregates, except for the presence of osmium black precipitate (Fig. 23). Amorphous black material surrounded the aggregates and was deposited between the cells. Electron-dense material was also present in normal-sized and swollen granules in many platelets. Connections between channels and granules and direct communication between canaliculi and the surrounding plasma were evident.

Amorphous precipitate was not present outside the thrombin-activated cells from samples of washed platelets resuspended in the presence of EDTA, and aggregates were absent (Figs. 24-26). The platelets, however, revealed the same physical changes observed in thrombin-aggregated cells from PRP. Many granules were stained intensely by tannic acid-osmium. Other granules were swollen and their content of amorphous stained material appeared diluted. Channels of the OCS were also delineated by electron-dense stain. Some channels were tortuous and narrow and contained little tannic acid. Others were filled by electron-dense material and widely dilated (Fig. 24). Communications between granules and OCS channels were evident in many platelets (Figs. 24-26). The connection appeared to foster swelling of the granule and dilatation of the channel so that recognition of the site of fusion was often obscured. More than one granule was frequently in communication with the same OCS channel (Fig. 25). This relationship often resulted in extensive dilatation of the OCS and granules fused to it. Occasionally, channel openings onto the surface were dilated, but usually remained constricted as in resting platelets.

Communications between channels of the OCS and surface membrane in washed platelets exposed to thrombin in the presence of EDTA were identical in appearance to those observed in thrombin-aggregated cells from C-PRP. Some channels were dilated immediately under the cell membrane, while others remained narrow for considerable distances in the cytoplasm. Many were connected to other canaliculi and to granules. Occasionally a single channel opened in more than one place onto the

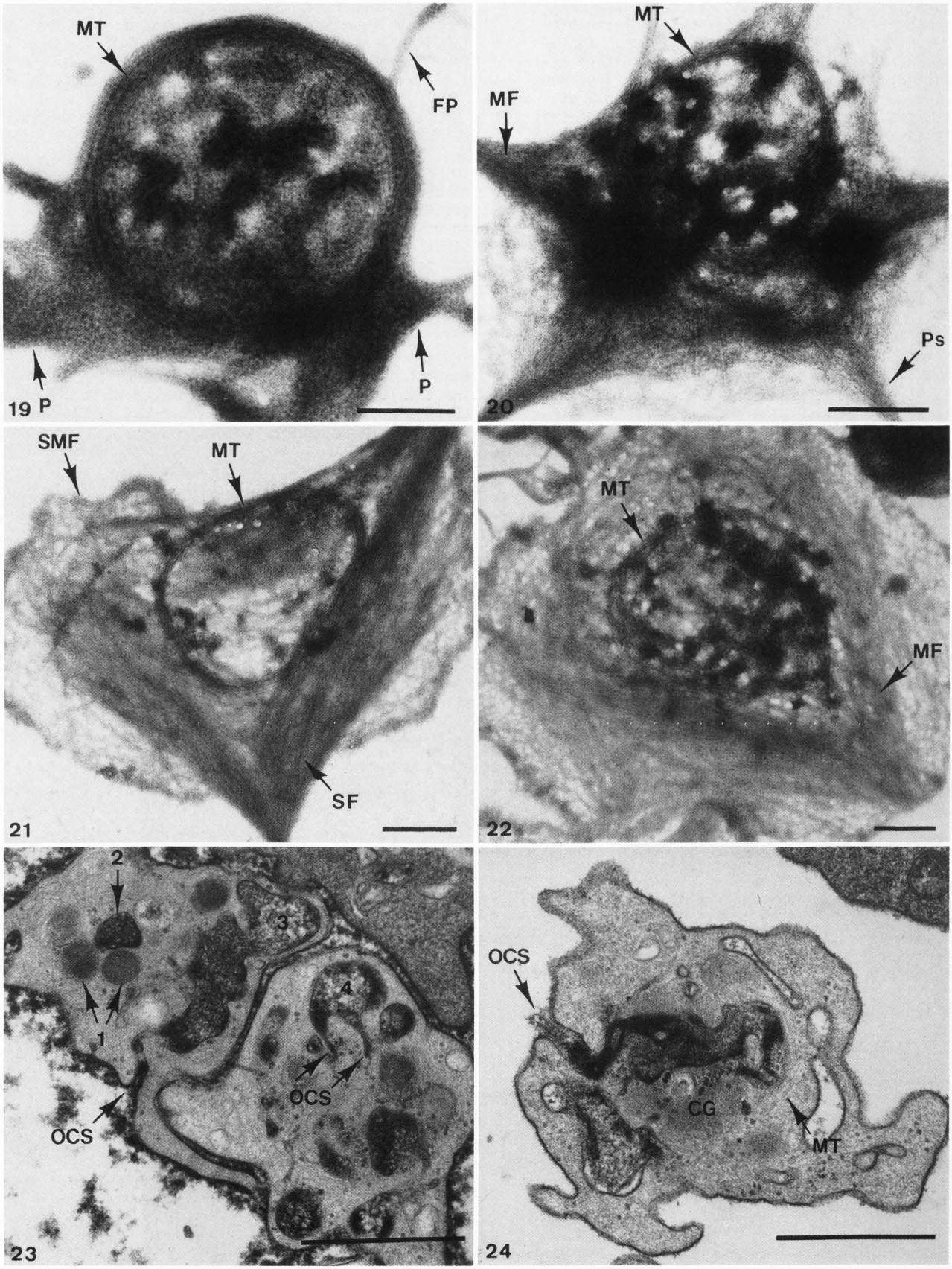
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Figure 19. Whole mount cytoskeleton of dendritic platelets. The discoid cell is manifesting early signs of stimulation by the foreign surface. Protuberances (P) of cytoplasm and filiform processes (FP) extend beyond the microtubule coils (MT). The protuberances and cytoplasm are filled with a mat-like network of short actin filaments. Bar = 1 μm.

Figure 20. Cytoskeleton of spread dendritic platelet. Surface stimulation has caused this cell to extend long, relatively rigid pseudopods (Ps). The spike-like extensions are filled with parallel bundles of actin microfilaments (MF). A loosely interconnected network of randomly arranged actin filaments fills spaces between the spike-like pseudopods. The circumferential microtubule (MT) is constricted and some of its free ends radiate into the cytoplasm. Bar = 1 μm.

Figure 21. Spread platelet cytoskeleton. The platelet has spread out into a thin film on the grid surface. Actin is organized into submembrane filaments (SMF) forming the cell border and into bundles in the cytoplasm resembling stress fibers (SF). Filaments of actin are also dispersed at random in clearer areas of the cytoplasm. The microtubule (MT) coils are constricted toward the cell center. Bar = 1 μm.

Figure 22. Spread platelet cytoskeleton. Coils of the circumferential microtubule (MT) are constricted in the cell center. Actin microfilaments (MF) form the cell border and are organized in concentric layers around the MT coil. Bar = 1 μm.

Figure 23. Human platelet secretion. Platelet aggregate form sample of C-PRP exposed to 1 U/ml of thrombin for 3 minutes. Clumps of tannic acid-osmium stained fibrinogen are evident around and between the aggregated cells. Some granules (1) are unstained while others (2) show a strong reaction. Another granule (3) communicates through dilated with narrow segments of the open canalicular system (OCS) with the surface membrane on the other side of the platelet. A fourth granule (4) appears to communicate with two segments of the OCS in an adjacent platelet. Bar = 1 μm.

Figure 24. Human platelet secretion. Platelet from washed suspension exposed to thrombin at a concentration of 5 U/ml for 1 minute in the presence of EDTA. A channel of the open canalicular system (OCS) filled with granule contents follows a tortuous course from one side of the centrally concentrated contractile gel (CG) and constricted ring of microtubules (MT) to an opening on the opposite side. Tannic acid osmium stained material appears to have been fixed in the process of extrusion from the opening. Bar = 1 μm.

surface membrane of an activated platelet (Fig. 26). The electron-dense material present in channels frequently appeared to extrude into the surrounding medium.

The fine structure of bovine platelets was described previously by Myers et al[33] and by Zucker-Franklin and her colleagues.[75] Bovine platelets are similar to human cells in many respects. Like platelets from man, cattle platelets are discoid in form and their lentiform appearance is supported by a circumferential microtubule (Fig. 27). Bovine platelets contain the granules, dense bodies and microchondria also found in human cells.

There are, however, significant differences between human and bovine platelets. Cattle cells are slightly smaller, contain fewer dense bodies and their cytoplasm is dominated by granules two to three times larger than those in human platelets. Many of the large granules are spindle-shaped and often appear to contact the inside of the surface membrane (Fig. 28). An extensive network of tortuous channels communicating with the cell surface and referred to as the open canalicular system (OCS) in human platelets was not found in bovine cells. This confirmed work showing that cationized ferritin, an electron dense tracer, which easily entered the channel system of human platelets, but did not appear in bovine platelet cytoplasm.[75] Replicas of freeze-fractured bovine platelets also failed to reveal the typical pits formed by channels of the OCS communicating with the surface of platelets from man.

Thin sections of thrombin-stimulated but not aggregated, bovine platelets revealed additional differences in their response compared to their human counterpart. Spike-like pseudopods extending from shape-changed bovine platelets were thinner and straighter than those formed on activated human platelets. Occasional microtubules are evident in human platelet pseudopods, but most surface projections lack the polymer. Almost every pseudopod extending from bovine platelets contained one or several microtubules interspersed with microfilaments. Microtubules were also present in the cytoplasm of activated bovine platelets. However, instead of being organized in constricted rings around centrally concentrated organelles, the tubules were irregularly dispersed in bundles or loose arrangements. Granules in most platelets were peripherally oriented near the cell membrane, rather than concentrated in cell centers.

Although tannic acid failed to stain channels of the OCS or granules in resting bovine platelets, it did so in thrombin activated cells. Tannic acid appeared to react selectively with some product in platelet granules resulting in a heavy deposit of osmium black. The electron dense stain appeared to reach granules through connections between the organelles and the cell surface (Fig. 29). The mechanism for development of the openings permitting penetration of tannic acid into granules was not clear. However, it must be related to the action of thrombin because connections were not evident in control platelets.

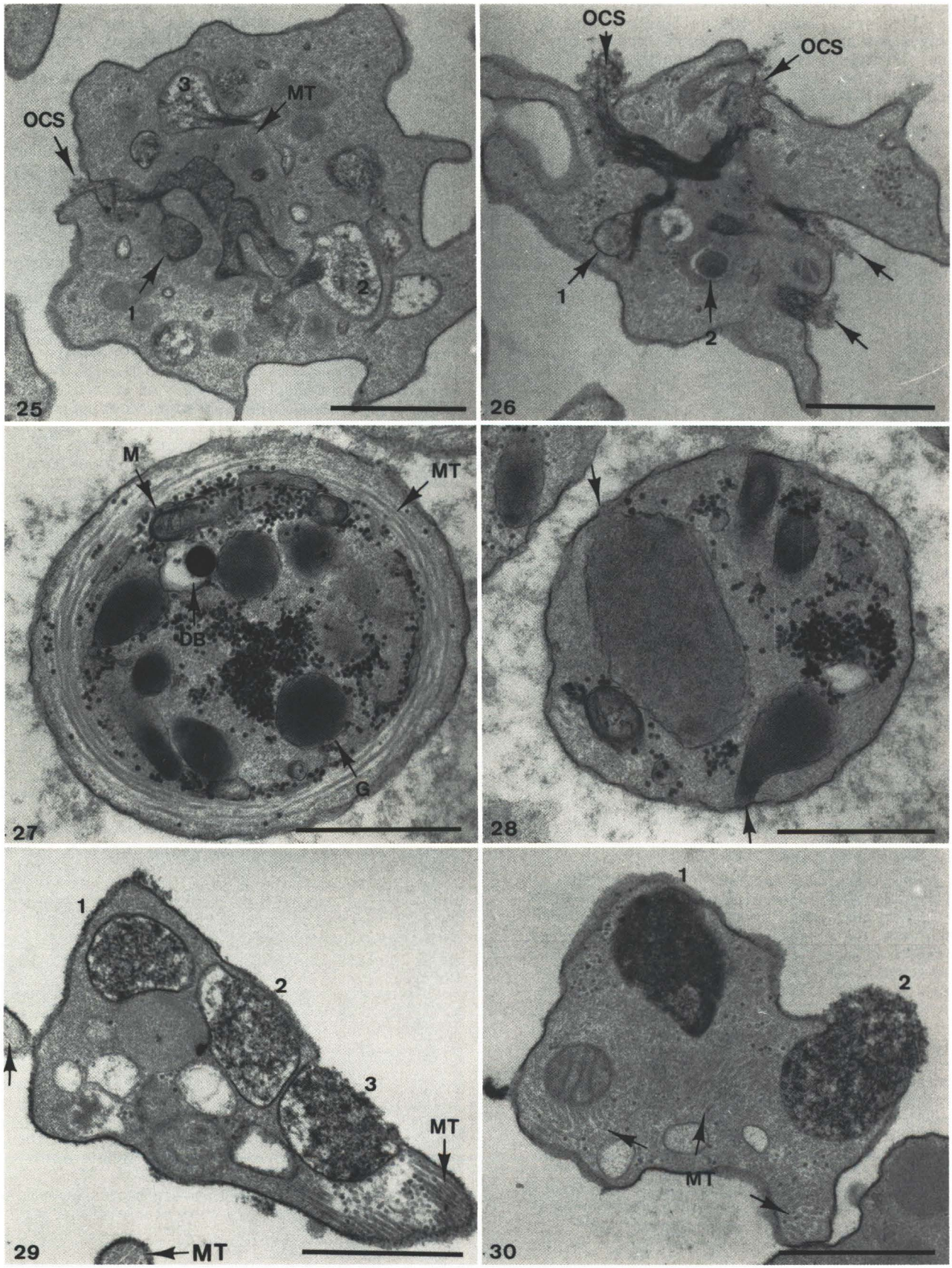
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Figure 25. Human platelet secretion. EDTA washed platelet exposed to 3 U/ml of thrombin for 5 minutes. Stained (1) and swollen (2) granules are directly connected to the channel filled with material reacting with tannic acid-osmium. Other swollen granules, such as 3, are connected to different channels of the open canalicular system (OCS) and pass out of the plane of section. Microtubules (MT) are constricted into the central mass of contractile gel. Bar = 1 μm.

Figure 26. Human platelet secretion. Platelet from sample of EDTA washed platelets exposed to thrombin at 3 U/ml for 1 minute. Electron-dense material is in the process of extrusion from four different sites (▲) on the surface of this cell. One of the channels inside the cell communicates with two different openings of the open canalicular system (OCS) and with a granule (1). Other granules (2) are not stained by tannic acid-osmium. Bar = 1 μm.

Figure 27. Resting bovine platelet. Thin section of discoid bovine platelet resembles the human cell closely (see Figure 2). However, it lacks channels of the open canalicular system. The disc-like form is supported by a circumferential microtubule (MT). Granules (G), dense bodies (DB) and mitochondria (M) are evident in the cytoplasm. Bar = 1 μm.

Figure 28. Resting bovine platelets. Two large granules in this cell appear to be in physical contact with the surface membrane (▲). Bar = 1 μm.

Figure 29. Bovine platelet secretion. Thrombin-activated platelet (1 U/ml, 3 minutes). Little evidence of shape change is apparent in this cell. Microtubules (MT) are still evident at the platelet periphery and adjacent pseudopods. One granule (1) stained by tannic acid appears unconnected to the cell surface, another organelle (2) is very close to it and a third (3) is open to the surrounding medium. Serial sections of this cell (not shown) reveal that organelles 1 and 2 are also directly connected to the platelet surface. Bar = 1 μm.

Figure 30. Bovine platelet secretion. Thrombin-activated platelets (5 U/ml, 1 minute). The microtubules (MT) in this cell are in three separate groups. One granule (1) is filled with stain and appears unconnected to the cell surface. However, it must be connected above or below the plane of section to the surface membrane in order to take up tannic acid. Another granule (2) is connected to the cell surface and its contents appear to be in the process of extrusion. Bar = 1 μm.

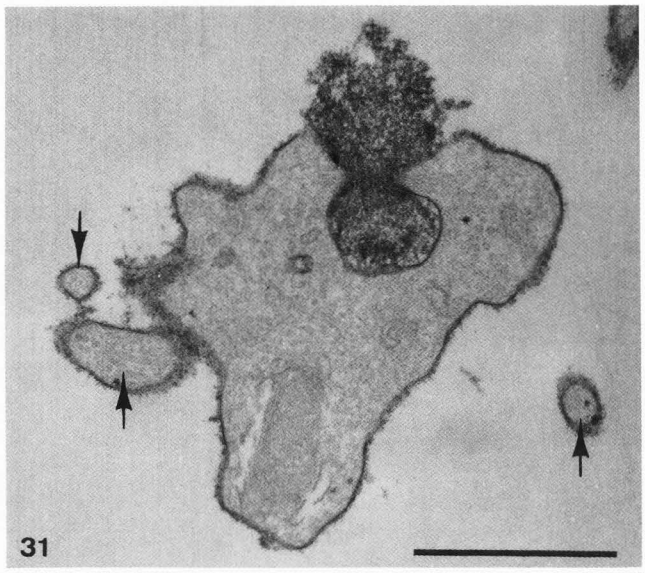
The discharge of stained granule contents from thrombin activated platelets appeared to follow two routes. Most of the granules close to the surface fused with the membrane and secreted directly to the outside medium (Figs. 30, 31). Some examples suggest that granule products are actively extruded. Internal contraction leading to formation of dense spots of contractile gel in thrombin-activated bovine platelets may provide the force for expulsion.

In addition to demonstrating the interaction between granules and the surface membrane in thrombin-activated bovine platelets, tannic acid staining also revealed development of a primitive channel system in activated cells. In thin sections of tannic acid-stained, bovine platelets, electron dense product penetrated into fragile channels extending for variable distances into the cytoplasm. Communications between stain-filled channels and granules were easily identified (Fig. 32). The channels appeared fragile and lacked the tortuosity and extensive distribution of the OCS in human platelets.

Thus, the difference in the mechanism of secretion in bovine compared to human cells is not as great as it might at first seem. Bovine platelets do not have a well-defined OCS. As a result, they use the surface membrane as the primary route for discharge of products from secretory granules. Human platelets have an extensive system of internalized surface membrane formed into channels of the OCS. It is used as the primary route of secretion in human cells. Bovine platelets can develop primitive canaliculi following activation and granule products can leave the cell through these conduits. Yet it employs the cell surface as a preferential route for exocytosis. The basis for the species variations in platelet structure resulting in different preferred routes for secretion of granule products remains unknown. However, it is clear that there are significant differences in bovine and human platelet shape change, pseudopod formation, spreading on surfaces and internal transformation. Differences, therefore, in the mode of platelet secretion are not as surprising as they might seem.

Platelet-Vessel Wall Interaction

The knowledge gained in studies of platelet activation in suspension or on foreign surfaces provides insights into platelet-vessel wall interaction.[3] Exposure of subendothelial connective tissue is the critical factor in causing the hemostatic reaction. Discoid platelets adhere almost immediately to the site of injury (Fig. 33). In order to resist the shear force of flowing blood, particularly in the arterial circulation, the platelets must become sticky and remain that way. They accomplish that by rapidly extending pseudopods and spreading out on the denuded surface (Fig. 34). The result is an initial monolayer of platelets attempting to replace the lost endothelial covering. Additional platelets adhere to the monolayer of spread cells and to each other to form aggregates (Fig. 35). The monolayer on a denuded vascular surface is similar to the platelet reaction to other foreign surfaces, and the



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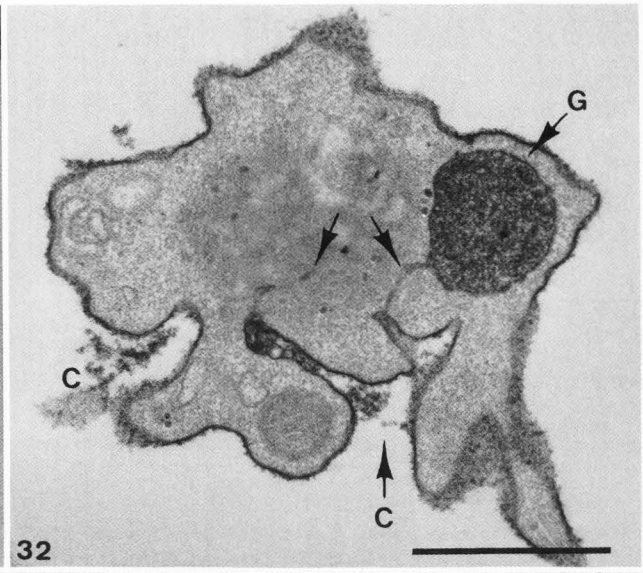

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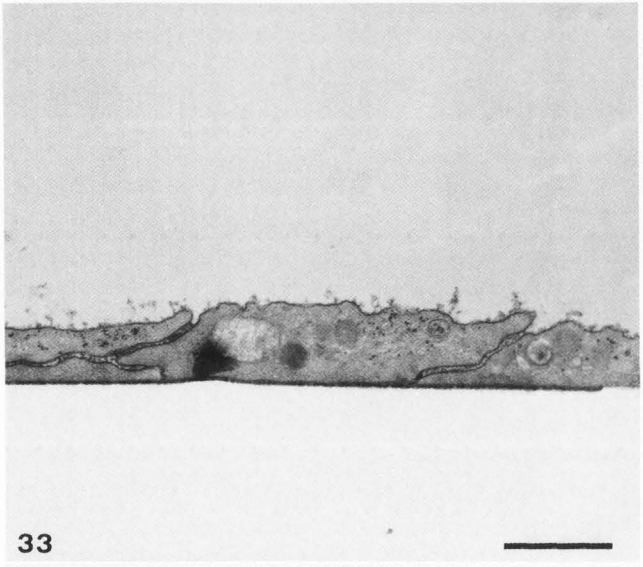

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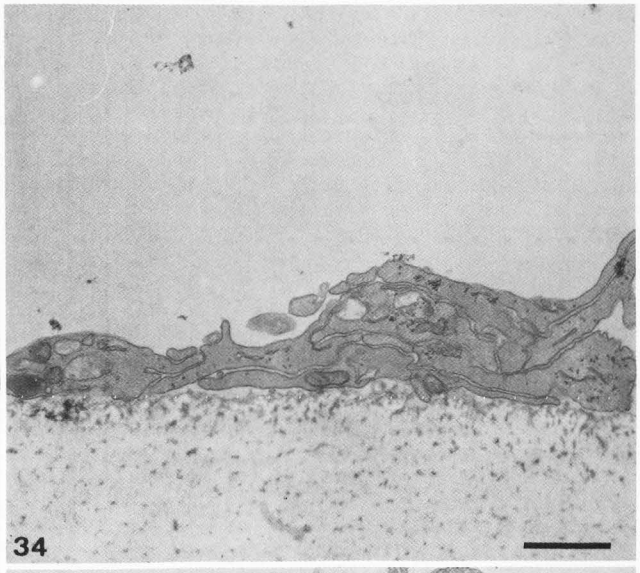

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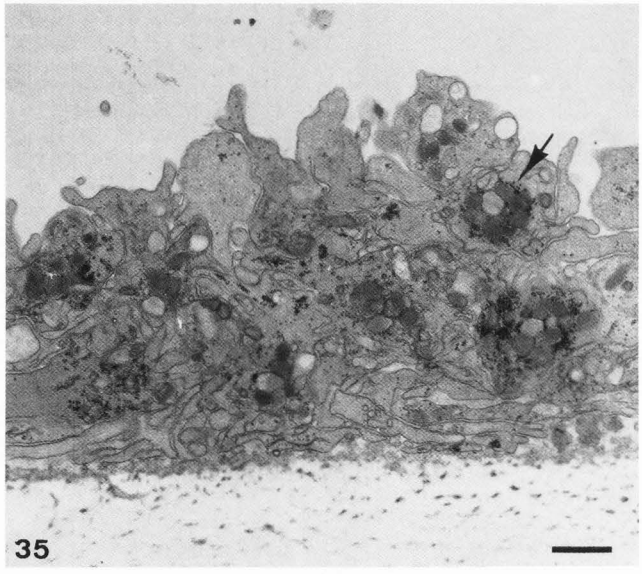

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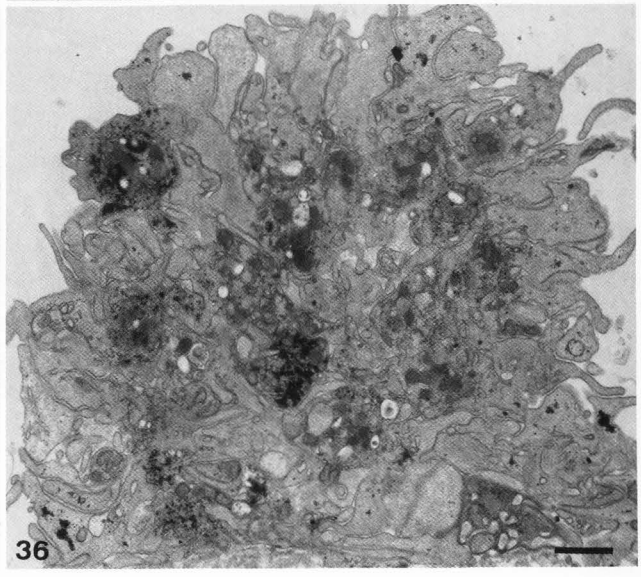

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Figure 31. Thrombin-activated platelet (5 U/ml, 1 minute). Every pseudopod (▲) extending from this platelet or adjacent cells contains microtubules. A granule filled with tannic acid stained material appears to be in the process of discharge from the platelet. Bar = 1 μm.

Figure 32. Thrombin-activated platelet (5 U/ml, 1 minute). Several channels (C) or empty granules have scalloped the cell membrane. Fine canaliculi (▲) stained by tannic acid extend from the surface deeper into the cytoplasm. One of them connects to a stain filled granule (G). Bar = 1 μm.

Figure 33. Platelet-surface interaction. Platelet rich plasma was allowed to settle on a polydysine-coated glass slide. Under these conditions the platelets form a monolayer of discoid cells. Physical changes are minimal. Bar = 1 μm.

Figure 34. Platelet-vessel wall interaction. Endothelial cells were removed from rabbit aorta, the denuded segment inverted and drawn onto the rod of a Baumgartner chamber.[3] Blood flowing over the surface at a shear rate of 800 sec^{-1} has deposited a near monolayer of platelets. Under these conditions the cells show signs of extensive spreading and interaction of pseudopods. Bar = 1 μm.

Figure 35. Platelet-vessel wall interaction. Platelets from flowing blood have formed an aggregate on a denuded vessel segment. Interactions between aggregated platelets appear identical to those occurring in cells stimulated in suspension, including the process of internal transformation (▲). Bar = 1 μm.

Figure 36. Platelet-vessel wall interaction. The aggregation of the platelets on this denuded segment of rabbit aorta has resulted in a layer of more than 5 microns in thickness. As a result it impinges on blood flow and has become a thrombus. Bar = 1 μm.

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aggregates resemble platelet-platelet interactions in suspension. As the size of the aggregates accumulating at sites of damage continue to grow, they become potentially occlusive and are referred to as thrombi (Fig. 36). Fibrin strands are commonly present. Thus all features of the platelet response observed in suspension and on glass and grids are incorporated into the reaction with injured blood vessels.

Platelet-Fibrin Relationship

Interactions between fibrin and platelets are essential for sealing off sites of vascular injury. In the past most of the work on platelet-fibrin associations was done on clots fixed under isotonic tension. The mass of platelets and fibrin squeezed out serum and red blood cells as it tightened on itself. Unfortunately, the collapse of structures during contraction and swelling of extruded pseudopods made it difficult to identify specific features of this unusual muscle system. Recently, it has become possible to study platelet-fibrin associations in clots allowed to contract under isometric conditions.[9] Platelet clots are prepared in the form of long cylinders and hung in physiological baths, just like muscle strips. The cylindrical clot is tied to the bottom and attached under tension to a strain gauge at the top. In this manner, force generation can be measured without clot shortening. Preparation of clots at various intervals during tension development has permitted detailed analysis of the platelet-fibrin relationship. Longitudinal sections reveal many long fibrin strands oriented like cables in the axis of tension. Platelets and platelet clumps are spindle shaped and also oriented in the long axis. Pseudopods and platelet bodies are intimately associated with fibrin strands. The cells appear to have spread out on fibrin strands, much as they do on injured vascular surfaces or glass slides (Fig. 37). Other platelets attach to the cells spread out on fibrin to form aggregates. Platelet aggregates contract on the fibrin strands and each other. As a result, fibrin forms twisted masses in aggregate centers and oriented strands in the axis of tension. Bundles of microfilaments and microtubules inside the contracting platelets (Fig. 38) spread out on fibrin are also oriented in the axis of force generation. Thus the interaction of platelets with fibrin in clots under isometric tension is much like that of platelets on other surfaces, except that the spreading and contraction are primarily in a single axis.

Discussion

The overview presented above has described a few of the current concepts regarding platelet structural physiology. Time and space do not permit us to go further, and the reader is urged to consult more extensive reviews of the subject for additional information.[74] Here we have emphasized certain aspects of platelet structure in resting cells and following activation. In particular, we have sought to demonstrate changes in the cytoskeleton and the fate of secretory organelles in human and bovine platelets following exposure to agonists in suspension, stimulation by surface contact or during the process of clot retraction.

The purpose in focusing on secretion was to try to clear up the current confusion regarding fundamental mechanisms involved in the platelet release reaction. Many years ago Behnke demonstrated that human platelets and cells from other species possessed a tortuous system of canalicular spread throughout the cytoplasm and connected to the cell surface.[4] Freeze-fracture and electron dense tracers revealed that channel openings tended to be clustered, rather than randomly dispersed, and were uniform in diameter. Channels were connected to each other just under

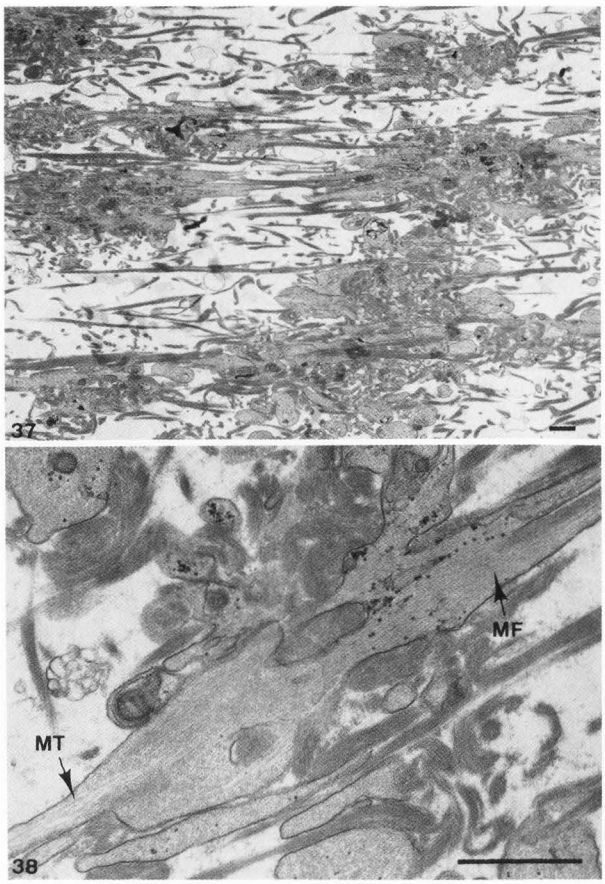

Figure 37. Clot retraction. The clot shown here was fixed at maximum tension during contraction under isometric conditions. Platelets and platelet pseudopods are oriented together with fibrin strands in the axis of tension. Bar = 1 μm.

Figure 38. Clot retraction. A higher magnification photograph of an isometrically contracted clot. The platelet body and pseudopods stretch out in the axis of tension. Microtubules (MT) and microfilaments (MF) inside the platelet body are also aligned. The organization strongly resembles a syncitium of smooth muscle cells. Bar = 1 μm.

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the cell surface and at other points in the cytoplasm. The result was a spider web of interconnected channels reaching from one side of the discoid platelet to the other which Behnke named the surface connected or open canalicular system (OCS).[48] Other investigations demonstrated that channels of the OCS often formed interwoven associations with elements of a second system, the dense tubular system (DTS), derived from rough endoplasmic reticulum of the parent megakaryocyte.[64] The membrane complexes formed by close associations of DTS and OCS channels resembled the relationships of transverse tubules and sarcotubules in embryonic muscle.[65]

Incubation of platelets with particles visible in the electron microscope revealed that thorium dioxide and small latex granules were taken up into channels of the OCS and transferred to intact granules without loss of platelet discoid shape.[51,60] Larger particulates entered the channels, but could also stimulate a process resembling phagocytosis at points on the surface away from channels.[50,76] This is not surprising since large particles represent just another surface for the platelet to react on, as with the grids or subendothelium shown here. The critical point in the older studies pertinent to the release reaction was that channels of the OCS were open in living platelets and could serve as conduits for substances to travel from plasma to secretory organelles in the cytoplasm.

Studies of internal transformation following exposure of platelets to potent agonists causing secretion had shown that storage granules are concentrated in cell centers and do not move to the surface membrane of the cell for discharge.[63,67] It seemed reasonable, therefore, to suggest that the OCS, as an extension of the surface membrane reaching the centrally concentrated granules, might serve as a conduit for secretion. Studies with electron dense stains demonstrated that channels of the OCS remained open before, during and after extrusion of granule-based secretory products, and portions of the granules were identified in channels of activated platelets.[62]

Evidence favoring an alternative secretory pathway involving fusion of centrally concentrated granules to each other, formation of sealed vacuoles filled with substances from the organelles, migration to the surface, attachment to the cell wall and discharge of contents to the outside[21,47] was not encountered in our early work or in more recent studies.[57,71] The possibility that centrally concentrated granules might fuse with each other at an early stage in the secretory process has been attractive for many years and was suggested in early investigations.[56] That granules can fuse with each other has been demonstrated in studies of platelets from individuals with inherited and acquired platelet disorders[30,66,70] Giant granules, probably resulting from fusion of normal-sized granules, were frequently present in abnormal cells.

More concrete evidence has been provided by investigations of platelet morphology during long term storage in vitro. Fusion of normal sized organelles to form giant granules is a common finding after storage of platelets under mildly alkaline conditions for 7-21 days.[49] A stage of fusion similar to that observed in long term stored platelets would seem essential in formation of closed secretion vacuoles. Although the appearance of vacuoles in thrombin-activated platelets has frequently been reported, none of the studies has shown that fusion of granules and development of a sealed, membrane-enclosed mass precedes development of empty sacs. Apparent fusion of two granules was reported in a previous study of platelet secretion employing tannic

acid.[45] In our experience, however, such an event is extremely rare.

The foregoing discussion does not mean that granules cannot form vacuoles or undergo fusion reactions. The granules in platelets treated with phorbol myristate acetate (PMA) undergo swelling and apparent loss of internal contents before the cells lose their discoid form.[66] However, the Swiss cheese appearance of PMA-treated platelets was not due to replacement of alpha granules by isolated vacuoles. Studies with electron-dense tracers indicated clearly that swollen granules in PMA-treated cells were continuous with dilated channels of the OCS.

The ability of individual granules to lose their internal contents was also shown in platelets treated with cytochalasin B before exposure to trypsin or thrombin.[52] Interestingly, the effects of these agents on platelet granules is dependent on the presence of the OCS. Preliminary studies in bovine platelets, shown to lack the OCS, have failed to demonstrate replacement of granules by vacuoles when treated with PMA or cytochalasin B and trypsin.

The basis for the current difference of opinion regarding the mechanism of platelet secretion probably stems from the ability of thrombin at high concentrations to labilize platelet granules at a very early step in shape change. As a result, water from the surrounding media can enter the granules and cause massive swelling before their contents have left the organelle or channels of the open canalicular system. Lower concentrations of thrombin produce the same process of internal transformation and secretion with slow loss of granule identity and absence of large vacuole formation observed with secretion-inducing concentrations of ADP, arachidonic acid, collagen and the ionophore, A23187.[67] As granule contents enter the OCS their membranes become part of the OCS. Thus a fully activated platelet has few identifiable granules or granule membranes. If a high concentration of thrombin is used, empty granule membranes may remain swollen and channels narrowed by the process of internal contraction. The swollen empty granules communicating with dilated or narrow channels might suggest large vacuoles resulting from granule fusion. However, they are the result of, rather than the mechanism underlying secretion.

The reason for demonstrating in this overview the alterations in platelet cytoskeletons after exposure to aggregating agents in suspension, following contact with foreign surfaces or denuded vascular segments and during the process of contraction in clots under isometric tension was to shed light on another area of controversy. Many workers believe that studies carried out on platelets in suspension have little reference to the hemostatic process. During hemostasis platelets interact with vascular surfaces by adhering and spreading out on the site of damage. Platelets also change shape when stimulated in suspension, but to some investigators the altered appearance does not resemble the spreading platelet involved in sealing off sites of vascular injury.

The observation that platelets not only contained contractile proteins, as Luscher had first pointed out,[29] but were muscle cells and contractile physiology dominated every phase of their response was put forward many years ago.[59] Time has served to confirm that thesis. However, the comparison to skeletal, cardiac muscle or smooth muscle can be overdrawn. The thin actin filaments are fully assembled and highly organized in these muscles, and myosin is often present in the form of thick filaments. Resting platelets do not have the assembled and organized actin and myosin filaments evident in the more conventional muscle cells.[16]

This observation is often construed as evidence that platelets are not muscle cells. Yet, it is the absence of an assembled actomyosin system that gives the platelets such a tremendous advantage for their role in hemostasis. Platelets may be called upon to remove bacteria from circulating blood or assist in prevention of tumor cell metastasis. They may interact in suspension to form emboli, or participate in graft rejection. Clearly, attachment to and spreading out on denuded vascular surfaces is not the only manifestation of the platelet response in which its contractile system is involved.

The ability of the platelet to assemble and organize its contractile system to meet whatever challenge requiring involvement is truly remarkable. Short pseudopods that appear on discoid platelets during normal flow in the microcirculation are due to focal assembly of actin near the platelet surface. Shape change leading to formation of spiky spheres in suspension represents the response to stronger stimulation. It is comparable to the dendritic stage on surfaces and is due to organization of actin filaments into parallel bundles. Spreading is due to assembly of actin into a peripheral weave of parallel filaments that force the margin of the cell to expand.[27] Spreading is the characteristic response on flat surfaces, but platelets often interact with irregular sites of damage. As a result the margin may be irregular, rather than circular in configuration. Parallel bundles of actin develop in the cytoplasm of such cells, and may serve as stress fibers to adapt and hold the platelet to the irregular conditions.[69] Formation of a concentric weave of actin filaments interacting with the marginal microtubule constrict the coils into tight rings around centrally concentrated organelles.[15,58] The organization of actin filaments and microtubules in platelets of clots under isometric tension is similar to that of conventional muscle cells.[9] The platelets are spread out in the form of spindles on fibrin strands in the axis of contraction. This ability of the platelet to assemble actin into filaments and organize them in a manner which will best adapt the cell to the job required is almost unique among the various types of muscle cells.

In summary, this overview has sought to clarify aspects of platelet secretion and provide an explanation for the seemingly complex organization of the platelet contractile system.

Marion Barnhart's beautiful investigation with the scanning electron microscope serves as an excellent background for the information presented here. I hope by peeling back the membrane of the activated platelet to see the world inside that we have added to her work.

Acknowledgements

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Discussion with Reviewers

M.R. Buchanan: What is the difference on aggregation versus adhesion of platelets in Bernard-Soulier syndrome (BSS) and thrombasthenia?
Author: The question is more complex than it appears. Thrombasthenic platelets are usually present in normal numbers, and do not differ in size or appearance from normal platelets. The defect in thrombasthenia is primarily due to a deficiency or absence of a surface membrane glycoprotein complex, glycoprotein (GP) IIb - IIIa. Exposure of GPIIb-IIIa following exposure of platelets to aggregating agents results in binding of fibrinogen, development of platelet stickiness and aggregation. Absence or marked deficiency of the GPIIb-IIIa complex results in failure of thrombasthenic platelets to bind fibrinogen become sticky or aggregate.

Patients with BSS are thrombocytopenic and their platelets are larger than normal. As a result of increased size, BSS platelets may appear abnormal. However, their ultrastructural features, except for size, are similar, if not identical to control cells. BSS platelets are deficient in glycoprotein Ib, GPV and, possibly, GPIX. GPIb is the receptor for von Willebrand factor (vWF). Absence or marked deficiency in GPIb results in failure of BSS platelets to adhere to damaged vascular surfaces due to the failure to bind vWF. Platelet GPIb is not required for aggregation. As a result, the response of BSS platelets to most aggregating agents is similar to that of normal cells. However, absence of the receptor for vWF makes them anergic to ristocetin or bovine fibrinogen, and the decrease or absence of GPV causes a diminished response to thrombin.

Thrombasthenic platelets have normal amounts of GPIb in their exterior coats, can bind vWF and should be able to adhere normally to damaged blood vessels. They do form a monolayer when thrombasthenic blood is perfused in a Baumgartner chamber over denuded rabbit aorta segments, but fail to spread, form platelet aggregates or develop thrombi.[1] Thus, the absence of a single glycoprotein or glycoprotein complex is not fully compensated by the presence of another in hemorrhagic disorders.[2]

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M.R. Buchanan: Dr. White shows platelets interacting avidly with the underlying vessel wall structures as illustrated beautifully in Figure 33. This is well accepted dogma. But there is some recent evidence (which, in fact, is a rediscovery of the wheel since it was first published by Jorgensen and ignored in 1974), that the tissue immediately underlying the endothelium is not thrombogenic and in fact is as thromboresistant as the endothelial cell itself. An interesting question to initiate discussion of this issue, is, do platelets really stick to the basement membrane, if it really is exposed?
Author: Dr. Buchanan, I believe, is referring to the observation that basement membrane purified from renal glomeruli does not cause platelet adhesion or stimulate activation. As mentioned above, platelet thrombi attached to the subendothelium of damaged vessels eventually slough off under shear stress in circulating blood. Despite exposure of the underlying tissue, platelets do not adhere a second time to the exposed subendothelium. Yet, a second injury to the same site results in a more vigorous platelet response than develops after the first insult.

The studies we and others have done involve the use of deendothelialized segments of rabbit aorta. Interaction of platelets with these surfaces has provided much information about the fundamental mechanisms involved. It can be argued, as Dr. Buchanan intimates, that this model is artificial and does not answer the question he has posed. It may not. Restricting injury to endothelium so that deeper tissues are not involved is virtually impossible. Platelets respond rapidly to balloon catheter injury or to deendothelialized vascular segments, but never cover the entire damaged surface or form the same degree of response in all damaged areas. Dr. Buchanan's question would suggest that areas exposed to deeper injury react, while those that are only deendothelialized remain unresponsive to platelets. At present I am unable to answer this question on the basis of my limited knowledge or the literature on the subject. Perhaps the discussion Dr. Buchanan wishes to stimulate will shed some light.

M.R. Buchanan: In the paper, under the section headed "Platelet Shape Change" Dr. White makes the statement that exposure of platelets to aggregating agents, foreign surfaces or denuded blood vessel causes rapid activation. A number of questions that come to mind and should stir up a lot of debate and increase blood pressure are: (1) How much denuded blood vessel actually exists in the chronic evolving atherosclerotic patient? (2) Do platelets stick to endothelium? These questions arise from a growing interest in the interaction of blood cells with dysfunctional blood vessel wall endothelium, not denuded blood vessel walls.
Author: Dr. Buchanan does not ask simple questions. Precisely how much denuded blood vessel surface exists in chronic evolving atherosclerosis is unknown. The "response to injury hypothesis" of atherogenesis is widely held, and proposes that some form of damage to endothelial cells is the primary event in the initiation of atherosclerosis.[1] Injury, however, does not mean destruction. Fatty streaks, the earliest recognizable antecedent of the atherosclerotic plaque, are often covered by a thinned-out layer of

endothelium. Some change in the endothelial surface fosters interaction with monocytes and migration of these cells into the subendothelium. The mononuclear cells and proliferating smooth muscle cells fill with lipid and become swollen. Expansion forces the mass into the lumen and stretching may force endothelial cells apart. Exposure of subendothelium fosters platelet interaction initially but in time the platelet aggregates and thrombi slough, leaving the deendothelialized areas bare. Such areas may remain unreactive to platelets.

Proliferation of smooth muscle cells and fibroblasts together with lipid uptake can convert the primary lesions into fibrous or atherosclerotic plaques. Endothelium may be lost from such lesions, particularly in areas of high shear stress or ulceration. Platelets may interact with these lesions during development but disappear in time. Fracture of atherosclerotic plaques, however, can result in fresh bleeding and formation of occlusive myocardial ischemia and infarction followed by sudden death.

Do platelets, as well as monocytes, stick to damaged endothelium? They do in vitro and may in vivo in certain disease states such as thrombotic thrombocytopenic purpura.[2] Yet the relationship between platelet-endothelial cell interaction and the pathogenesis of atherosclerotic disease is not yet clear. Platelets are rich in growth-promoting factors, such as platelet-derived growth factor (PDGF), which stimulates smooth muscle cell and fibroblast proliferation. Their release into the subendothelium directly or by diffusion through damaged endothelial cells could promote atherosclerosis. Clearly, however, atherosclerosis is a multifaceted disease and the role of platelet-endothelial cell or subendothelial interaction in its development remains to be clarified.

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M.R. Buchanan: What is the teleological function of the platelet-fibrin contracting factor?

Author: Blood collected in glass tubes without anticoagulant will slowly form a solid gel. The interval required for sol-gel transformation is the clotting time. If the clot is loosened from the sides of the tube, it will contract into a solid mesh and serum will be squeezed from it. This process was already well recognized by Hewson[1] in 1772 and has been carefully studied by many investigators.[2]

Clot retraction, along with the bleeding time and clotting time, served as the only tests required to assess hemostatic capability before surgery for many decades. In view of our current state of knowledge, one can't help but wonder what we were testing.

Therefore, Dr. Buchanan's question is particularly appropriate. What is the ultimate purpose for platelet-fibrin interaction and contraction? Was it conceived in the original design when platelets were amoebocytes, and blood was a beautiful blue fluid that did not contain fibrinogen? Levin's[3] studies showed that amoebocytes can contract and squeeze out a granule-based protein that gels readily on contact with endotoxin. Interaction between the amoebocytes and protein gel may have resulted in contraction and sealing off sites of vascular injury in invertebrates.

Did platelet-fibrin interaction serve the same purpose in vertebrates? The long bleeding times found in patients with congenital afibrinogenemia and in thrombasthenia suggest that platelet-fibrinogen interaction is essential for normal hemostasis. It is curious, however, that bleeding problems are not usually as serious or frequent in congenital afibrinogenemia or thrombasthenic patients, as in individuals with chronic hemophilia. After all, the hemophiliac has platelets and fibrinogen; he just forms clots more slowly than normal persons.

Despite the red flags raised by experiments of nature, it would appear that platelet-fibrinogen interaction is important for development of platelet stickiness, even if it is not absolutely required. It appears to be involved in platelet-vessel wall interaction, formation of hemostatic plugs, contraction of the injured site and initiation of events leading to clot lysis.

Yet, is the in vitro phenomenon of clot retraction a true reflection of an in vivo event required for hemostasis, and if so, what is its ultimate purpose? The answer, I am afraid is both yes and no. Clot retraction in vitro is a reflection of the contractile properties of blood platelets and their ability to adapt muscle protein organization to serve hemostatic function. When performed under isometric conditions[4] a great deal can be learned about how platelets interact with a surface such as fibrin in order to exert force; how the cells interact with each other to form the equivalent of sarcomeres; and how the contractile elements assemble and organize in each platelet in order to operate efficiently as a muscle system. Thus, the teleology of the platelet is to act as a muscle cell, and the interaction with fibrin in retracting clots is just one reflection of the universal mechanism involved in platelet function.

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2. Luscher EF (1961) Retraction activity of the platelets: Biochemical and physiological significance. In: Blood Platelets (Johnson et al, Eds), Boston, Little Brown and Co., pp. 445-453.
3. Levin J (1967) Blood coagulation and endotoxin in invertebrates. Med. Proc. 26, 1701-1712.
4. Cohen I, Gerrard JM and White JG (1982) Ultrastructure of clots during isometric contraction. J. Cell Biol. 93, 775-787.

H. Feinberg: What evidence supports the view that high concentrations of thrombin labilize platelet granules such that water enters the granules and causes massive swelling? This would require that the granules in the resting platelet be maintained in a hyperosmotic state and that the granule-cytosolic barrier be impermeable to water.

Author: The suspicion inherent in the reviewer's question is correct. Specific information regarding the state of water and protein inside platelet alpha granules is not at hand to answer his question. Yet, considerable information is available from other sources. Platelet alpha granules and lysosomes are similar in many respects to specific granules, lysosomes and other storage organelles in leukocytes and in other tissues. Many of these organelles form by fusion of vesicles derived from the Golgi apparatus. Elegant studies have demonstrated the process of organelle formation in bone marrow precursors of circulating neutrophils. Studies on megakaryocytes are not quite so clearcut, but show a similar pattern. Vesicles containing minute amounts of protein fuse together to form the early lysosome or secretory organelle. During early stages the content of the organelles appears dilute in thin sections. Increasing maturity results in concentration of protein molecules in the organelles and disappearance of the peripheral clear appearance, most likely due to water. Thus the proteins inside lysosomes are concentrated relative to water and in a hyperosmolar state. The condition is common to virtually all storage organelles.

Many years ago de Duve used the term "structure linked latency"[1] to describe the anergic state of lysosomes inside unstimulated cells. It is just as appropriate for the non-lysosomal organelles in neutrophils, monocytes and platelets. Structure linked latency refers to the quiescent state of the organelle contents. They do not interact with cytoplasmic constituents, but remain isolated and concentrated within their membranes until they are labilized.

Labilization can occur in a variety of ways. Phagocytic vacuoles containing ingested bacteria interact and fuse with lysosomes in neutrophils.[2] Release of concentrated proteins from the organelles may cause some of the swelling observed in vacuoles with foreign organisms.

Platelets can form vacuoles after uptake of foreign particles. Latex spherules taken into channels of the open canalicular system may form concentrated masses, break down the channels separating them and fuse with lysosomes and granules.[3] They do not seal off, however, as phagocytic vacuoles in other cells usually do. Electron dense tracers and freeze fracture have shown vacuoles remain in continuity with the platelet surface through channels of the OCS.

The swelling of platelet granules, however, is more clearly shown by their response to chemical agents than by uptake of foreign particulates. Phorbol myristate acetate (PMA)[4] and 1-oleoyl-2-acetyl glycerol (OAG) cause platelet alpha granules to swell and lose their contents to the OCS, even before the cells lose their discoid shape. PMA and OAG are surface active agents and selectively perturb the structure linked latency of platelet organelles. The swelling appears due to the movement of water across the OCS-granule membrane barrier permeabilized selectively by the influence of the chemical agents.

Thrombin can also result in granule swelling, but low concentrations do not. Instead, low thrombin causes shape change, internal transformation and secretion of granule contents which are squeezed out of the cell through channels of the OCS. The sequential transformation resembles that produced by other platelet activating agents. Apparently the step-wise process permits the granules to be extruded before they can be converted into vacuoles.

High concentrations of thrombin stimulate a rapid platelet response. Granules fuse with channels and discharge their contents. Fibrinogen is released into the OCS and thrombin reaches the channels, since the coagulant protein is converted to thrombin.[5] Under these conditions both granules and channels become swollen, and resemble secretion vacuoles. The reason for their formation may be related to the rapid response to high dose thrombin. Activation under these conditions may trigger very rapid assembly of actin and maximum contraction before granule contents are extruded. As a result some of them may remain in the granules and OCS and add a significant concentration of protein molecules to attract plasma water to cause excessive swelling.[6]

1. de Duve C, Pressman BC, Gianetta R, Waltiaux R, and Appelmans F (1955) Tissue fractionation studies VI Intracellular distribution patterns of enzymes in rat liver tissue. Biochem. J. 60, 604-614.
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3. White JG and Clawson CC (1981) Effects of large latex particle uptake on the surface connected canalicular system of blood platelets: A freeze fracture and cytochemical study. Ultrastruc. Pathol. 2, 277-287.
4. White JG and Estensen RD (1974) Cytochemical electron microscopic studies of the action of phorbol myristate acetate on platelets. Am. J. Pathol. 74, 453-466.
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6. White JG and Krumwiede M (1987) Further studies of the secretory pathway in thrombin stimulated human platelets. Blood 69, 1196-1203.

H. Feinberg: Your view suggesting a parallel or alternative secretory pathway in bovine platelets exposed to high thrombin concentrations that is similar to a pathway (via the OCS) seen in human platelets is interesting. Would it also be reasonable to postulate that the vacuolar structures seen in human platelets after exposure to high thrombin levels are the result of the combining of granules and the secretion of their contents across the plasma membrane, rather than the swelling of individual granules and the subsequent release of their contents into the OCS?

Thus both types of platelets would be capable of utilizing alternative secretory pathways under special conditions, i.e., high thrombin levels.

Author: The alternative mechanism you have suggested for human platelet secretion has been proposed previously by others.[1] Ginsberg et al used immunofluorescence with monoclonal antibodies to granule products and electron microscopy to follow the secretory process in thrombin-stimulated human platelets. Their findings suggested that granules fused together in thrombin activated platelets to form closed sacs resembling secretion vacuoles. The sealed vacuoles appeared to migrate to the platelet surface, fuse to the membrane and discharge products to the exterior. The proposed hypothesis is essentially identical to the mechanism put forward by the reviewer suggesting that human platelets may share both of the secretory pathways observed in bovine cells.

Platelet granule fusion into a membrane enclosed sac subsequently released through the platelet surface represents a very attractive concept. One form or another of this hypothesis for human platelet secretion has been popular since the formation of the centrally concentrated granulomere in spread platelets was first recognized on glass slides. Early electron microscopic studies also suggested granule fusion in stimulated platelets, but did not identify the pathway for secreted products.

The proposed mechanism is not entirely limited to platelets, and, therefore, receives credence from nature. Neutrophil lysosomes, for example, fuse with phagocytic vacuoles containing bacteria to form closed sacs.[2] However, the membrane forming the phagocytic vacuole often retains connections to the surface membrane from which it was formed. As a result, enzymes derived from the fused lysosomes can find their way from vacuoles to the outside medium.

There are a number of problems with the mechanism of platelet granule release suggested by the reviewer. Neutrophils were chosen as a potentially supportive example because of their similarity during phagocytosis to the mechanism proposed for platelet granule secretion. Neutrophils are not secretory cells in the sense platelets are. Secretion of lysosomes into intracellular phagocytic vacuoles is meant to confine the enzymes around the ingested organisms and prevent exposure of the rest of the cell cytoplasm and surrounding tissue to toxic substances. Even though some enzymes may leak out through residual connections to the cell surface, this can hardly be looked upon as a mechanism fostering secretion. Also, it is noteworthy that neutrophil lysosomes and granules do not fuse with each other, only with the phagocytic vacuoles.

The first sign of platelet response to thrombin is not formation of swollen granules or secretion vacuoles. Thrombin stimulates rapid assembly of actin molecules into filaments which cause shape change and internal transformation. Secretory organelles are concentrated in platelet centers where they are enclosed by a tight-fitting web of microtubules and microfilaments. Secretion of granule products through the OCS or the development of swollen granules follow these events. If swollen granules were to fuse into sealed vacuoles destined for secretion, one would have to wonder how they can reach the cell surface to escape. The cytoplasm is filled with a forest of assembled polymers in the thrombin activated cell. Passive transfer of vacuoles to the surface would have to negotiate what appears to be an impenetrable barrier. Such a mechanism might serve to isolate digestion vacuoles in the neutrophil cytoplasm where secretion is undesirable. However, a major function of platelets is to secrete substances to the exterior of the cell in a controlled, but rapid manner. A system involving movement of granules to cell centers, fusion with each other to form sealed vacuoles, migration of the vacuoles through a maze of polymers to the surface, attachment to the membrane and extrusion of contents to the media would seem rather awkward for this mission to be accomplished.

PMA, OAG and thrombin do stimulate vacuole formation, though thrombin does so only at high concentrations. PMA acts primarily on the OCS-granule relationship in a way different than any other agent, except perhaps OAG. Most other agents stimulate platelet shape change, internal transformation and secretion without causing formation of large vacuoles. While formation of vacuoles can occur in platelets, its relationship to the secretory process is unknown. As mentioned in an answer to a previous question, it is possible that rapid assembly and contraction of actin filaments in thrombin stimulated platelets, combined with an overreaction between granules and channels of OCS could result in narrowing of connections between the OCS and cell surface. The net result would distend the channels and connected granules leading to the appearance the reviewer has proposed as a mechanism of secretion.

The data presented in this report and in two recent papers in Blood[3,4] present the concept developed in this laboratory. Bovine platelets lack an OCS and must use some other conduit as a major secretory pathway. On stimulation with thrombin, cytoplasmic granules in bovine platelets fuse directly with the cell surface and discharge their contents to the exterior. A few cells develop delicate channels linking some granules to the cell surface, but this appears to be a minor secretory pathway. In contrast, human cells have a well-developed OCS which serves as the exclusive pathway for release of products from granules during secretion. A clearcut demonstration that sealed vacuoles formed by granule fusion serve any significant role in human platelet secretion has not been observed in our studies.

1. Ginsberg MH, Taylor L, Painter RG (1980) The mechanism of thrombin-induced platelet factor 4 secretion. Blood 55, 661-668.
2. Zucker-Franklin D and Hirsch JG (1963) Degranulation of polymorphonuclear leukocytes following phagocytosis--an electron microscopic study. Blood 22, 824-836.
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J. Breton-Gorius: Although unknown, the reason for the different routes for secretion in two platelet species: human and bovine remains fascinating. It appears that the membrane complex of human platelets[1] which can be hypertrophied in several congenital and acquired disorders[2-6] is not essential for the function of bovine platelets. It would be of interest to see if some molecular differences exist between the two platelet species upon stimulation.

On Figure 5 which represents a platelet whole mount, not only dense bodies are electron opaque, but in some of them, a dense membrane surrounds them, at distance of the opacity or a long single or double tail are seen. What they represent? What is the aspect at the level of thin sections?

If there is an agreement for the opening of OSC in living platelets, the size of the opening is small in resting platelets and increases during the release reaction[7]. This narrow pore can easily be closed by the cell coat protein after fixation by aldehydes. Thus, if antibodies against platelet glycoproteins (Gp) are applied on fixed platelets, the immunogold labeling present on the cell membrane is absent from the OSC[8]. The same is true for the demarcating membrane system of megakaryocytes[9]. The proof that platelet Gp Ib or IIb-IIIa are present on the OSC is given by incubating living platelets with antibodies[10]. However, the size of gold particles is critical, and using large particles (40 nm) the labeling is inconsistent (unpublished data).

The observation of the transfer of thorium dioxide in the intact alpha granules[11] appears important due to the recent evidences that some proteins present in the alpha granules can be stored from the plasma. This is probably the case for immunoglobulins[12], while other proteins are synthesized by megakaryocytes and packaged in the alpha granules[13-16]. Another tracer, the horseradish peroxidase, can also be incorporated in the alpha granules of megakaryocytes after its intravenous injection in guinea pig[17]. Thus the alpha granules represent a very special class of organelle where some proteins, but not all, can be stored from the plasma with those synthesized by megakaryocytes.

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Author: The electron dense membrane enclosing some opaque organelles in Figure 5 is a frequent finding in platelet whole mount preparations. It is also apparent in thin sections of glutaraldehyde-osmium fixed platelets as a thin dense layer on the inner half of the membrane enclosing some dense bodies. It may be the substance stained by the uranaffin reaction.[1] The dense material appears identical in thin sections to the opaque mass forming the dense body itself. The opaque material forming single or double, whip-like tails are extensions of the dense body mass. They are readily apparent in thin sections and have been discussed in previous reports.[2-4]

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